

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



F2

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁶: C12Q 1/68</p>	<p>A1</p>	<p>(11) International Publication Number: WO 97/40187 (43) International Publication Date: 30 October 1997 (30.10.97)</p>
<p>(21) International Application Number: PCT/GB97/01107 (22) International Filing Date: 21 April 1997 (21.04.97) (30) Priority Data: 9608133.6 19 April 1996 (19.04.96) GB (71) Applicant (for all designated States except US): GEMINI INTERNATIONAL HOLDINGS LIMITED [MC/MC]; Le Regina, 13-15, boulevard des Moulins, MC-98000 Monaco (MC). (72) Inventors; and (75) Inventors/Applicants (for US only): SPECTOR, Timothy, David [GB/GB]; St. Thomas's Hospital, Lambeth Palace Road, London SE1 7EH (GB). KEEN, Richard, William [GB/GB]; St. Thomas's Hospital, Lambeth Palace Road, London SE1 7EH (GB). (74) Agent: SCHLICH, George, William; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).</p>		<p>(81) Designated States: AU, CA, JP, MX, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: DIAGNOSTIC METHOD AND APPARATUS (57) Abstract A method of diagnosing a disease associated with a genetic polymorphism in a vitamin D receptor gene comprises determining the genotype of said vitamin D receptor gene in an animal. The method can be used to diagnose predisposition or susceptibility to osteoarthritis. Compositions for said diagnosis are provided. Methods of treatment of osteoarthritis are provided, comprising identifying an individual having a predisposition or susceptibility to the disease and subsequently treating that individual.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakhstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

DIAGNOSTIC METHOD AND APPARATUS

This invention relates to diagnostic method and apparatus based upon a polymorphism in a vitamin D receptor gene. More specifically, this invention relates to a method for diagnosis of pre-disposition or susceptibility to certain disease states, by screening for the presence of this polymorphism. The invention also relates to compositions for screening for the polymorphism.

Osteoarthritis, or degenerative joint disease as it is also known, is one of the most common types of arthritis. It is characterised by the breakdown of the joint's cartilage, causing bone to rub against bone causing pain and loss of movement. Osteoarthritis can range from very mild to very severe and most commonly affects middle-aged and older people. It affects hands and weight-bearing joints, such as the knees, hips, feet and back.

Although age is a leading risk factor, at present the aetiology and pathogenesis of this condition remain largely unknown. Many environmental factors and other independent conditions have been associated with osteoarthritis, including obesity, previous injury and/or menisectomy, knee bending occupations, smoking, sex hormones, gynaecological disorders and other metabolic factors. Obesity may lead to osteoarthritis of the knees. Also, people with injuries to the joints because of sports, repeated movements, or accidents may be at increased risk of developing osteoarthritis.

At present accurate diagnosis of osteoarthritis is in general possible only when the disease has progressed significantly. Physicians can do little more than make a diagnosis of osteoarthritis based on a physical examination and history of symptoms. X-ray is typically used only to confirm diagnosis.

A recent study concluded that weight loss in middle-aged people who are overweight can significantly reduce the risk or even prevent knee osteoarthritis from developing. In such cases an ability to improve accuracy of diagnosis of predisposition or susceptibility to osteoarthritis would be of distinct advantage.

- 2 -

It is an object of the present invention to improve diagnosis of osteoarthritis. It is a particular object of the invention to provide a method of diagnosis of predisposition or susceptibility to osteoarthritis. A further object is to provide, following such diagnosis, a method of preventative or palliative therapy for osteoarthritis before the disease becomes significantly established. Another object is to provide means for diagnosing predisposition or susceptibility to osteoarthritis.

In a first aspect, the invention provides a method of diagnosing a disease associated with a genetic polymorphism in a vitamin D receptor gene in an animal predisposed or susceptible to said disease, said method comprising determining the genotype of said vitamin D receptor in said animal. The first aspect of the invention further provides a method of identifying an animal predisposed or susceptible to a disease associated with a genetic polymorphism in a vitamin D receptor gene, said method comprising determining the genotype of said vitamin D receptor gene in said animal.

According to an embodiment of the first aspect of the invention there is provided a method of diagnosis of predisposition or susceptibility to osteoarthritis comprising determining genotype of a vitamin D receptor gene of an individual. Typically, the method comprises determining whether an individual is homozygous or heterozygous for alternative versions of a vitamin D receptor gene. In an embodiment of the invention, the method of diagnosis is to screen for an individual at risk of a condition or disease such as osteoarthritis correlated with a vitamin D receptor gene polymorphism.

Polymorphisms of the vitamin D receptor gene are known and the invention is based upon the observation of a correlation between polymorphisms in the vitamin D receptor gene, specifically in exon 9 of this gene and in the region from exon 7 to the 3' UTR, and predisposition or susceptibility to osteoarthritis. The invention is of advantage in that by screening for the presence of the polymorphism it is possible to identify individuals likely to have a genetic predisposition or susceptibility to the disease. Typically, screening is achieved using *in vitro* techniques.

- 3 -

In an embodiment of the invention diagnosis is carried out by determining whether a fragment of the vitamin D receptor gene contains a cleavage site for restriction enzyme TaqI. An individual is typically then classified according to whether the fragment contains a cleavage site for TaqI, wherein possessing a fragment that contains the cleavage site correlates with decreased risk of predisposition or susceptibility to osteoarthritis.

Thus, a vitamin D receptor gene in which the TaqI cleavage site is absent can be classified as a risk polymorphism of the gene, that is to say a polymorphic variant of the gene that is correlated with increased risk of predisposition or susceptibility to osteoarthritis. As a human genome contains two vitamin D receptor genes, one on each of a pair of chromosomes, an individual can accordingly be found to be homozygous or heterozygous for the risk polymorphism, or to lack the risk polymorphism.

In a further embodiment of the invention the method comprises determining whether a fragment of the vitamin D receptor gene contains a cleavage site for restriction enzyme BsmI. An individual is classified according to presence or absence of the cleavage site, wherein being homozygous for absence of the cleavage site correlates with decreased risk of predisposition or susceptibility to osteoarthritis. In this embodiment, a polymorphism of the gene in which the BsmI cleavage site is present is a risk polymorphism of the gene.

In a still further embodiment of the invention, the method comprises determining whether a fragment of the vitamin D receptor gene contains a cleavage site for restriction enzyme ApoI. An individual's risk of predisposition or susceptibility to osteoarthritis is classified as increased when the gene contains a cleavage site for ApoI, and being homozygous for presence of the cleavage site indicates an individual likely to be at increased risk.

The method conveniently comprises amplifying a fragment of a vitamin D receptor to produce copies and determining whether copies of the fragment contain a cleavage

- 4 -

site for one of the restriction enzymes TaqI, BsmI or ApeI. The method optionally includes testing the vitamin D receptor genotype using more than one of these restriction enzymes, or all of them.

A suitable technique is to amplify the fragment using PCR techniques, producing copies of a fragment that is at least 500 base pairs in length, preferably at least 600 base pairs in length. It is preferred that the PCR primers are selected so as to amplify a region of the gene that is about 740 base pairs in length. PCR techniques are well known in the art and it would be within the ambit of a person of ordinary skill in this art to identify primers for amplifying a suitable section of exon 9 of the vitamin D receptor gene. PCR techniques are described for example in EP-A-0200362 and EP-A-0201184. In a further embodiment of the invention, the diagnostic method comprises analysis of the vitamin D receptor gene using single strand conformational polymorphism (SSCP) mapping to determine whether the vitamin D receptor gene is the risk or the non-risk form.

In a specific embodiment of the invention, described in an example below, diagnosis is carried out comprising amplifying a fragment of exon 9 of a vitamin D receptor gene, in particular a fragment that is about 740 base pairs in length. Alternatively, the method uses other methods of analysis of the genotype of an individual to determine whether the fragment contains a cleavage site for TaqI.

Suitable PCR primers are:

- 1: CAGAGCATGGACAGGGAGCAAG (3')
- 2: GCAACTCCTCATGGCTGAGGTCTC (3')

wherein the amplified fragment is about 740 base pairs in length. Using these primers, a fragment of DNA is amplified and tested for whether a TaqI cleavage site is present. The site is present for example where a ATT codon in exon 9 has been changed to ATC.

In further specific embodiments of the invention, the method comprises determining

- 5 -

genotype of a region of a vitamin D receptor gene from exon 7 to the 3' UTR, using either BsmI or Apol. The methods comprises amplifying the specified region and testing for presence or absence of a cleavage site for restriction enzyme BsmI or Apol presence of the polymorphism being the risk genotype.

A further embodiment of the method of the invention, for diagnosis of predisposition or susceptibility to osteoarthritis in an individual, comprises determining whether the individual possesses a risk related polymorphic version of a vitamin D receptor gene, a risk related polymorphic version of the gene being one that lacks a cleavage site for restriction enzyme TaqI, the method comprising

using PCR techniques to amplify a fragment of exon 9 of the vitamin D receptor gene to make copies of the fragment,

testing whether the copies contain a cleavage site for TaqI,

and thereby determining whether the individual is homozygous or heterozygous for a polymorphic version of the gene, and

diagnosing that individual's risk of predisposition or susceptibility to osteoarthritis as greatest if that individual is homozygous for the polymorphic version of the gene and least if that individual lacks the polymorphic version. In an example of the invention, the PCR primers:

1: CAGAGCATGGACAGGGAGCAAG

2: GCAACTCCTCATGGCTGAGGTCTC

are used to amplify a 740 base pair fragment of exon 9 of a vitamin D receptor gene.

As described above, in preferred embodiments of the first aspect of the invention, the method comprises screening a vitamin D receptor gene, and this screening is conveniently carried out by any one of a number of suitable techniques that are known in the art, and may be conveniently selected from amplification of a nucleic acid

- 6 -

sequence located within the vitamin D receptor gene, Southern blotting of regions of the gene and single strand conformational polymorphism mapping of regions within the gene. The genotype in that region is also optionally determined using hybridisation probes adapted selectively to hybridise with a particular polymorphism of the vitamin D receptor gene which is associated with predisposition or susceptibility to disease.

A probe used for hybridisation detection methods must be in some way labelled so as to enable detection of successfully hybridisation events. This is optionally achieved by *in vitro* methods such as nick-translation, replacing nucleotides in the probe by radioactively labelled nucleotides, or by random primer extension, in which non-labelled molecules act as a template for the synthesis of labelled copies. Other standard method of labelling probes so as to detect hybridisation are known to those skilled in this art.

According to a second aspect of the invention there is provided a method of diagnosis and therapy comprising diagnosing predisposition or susceptibility to osteoarthritis according to the method of the first aspect of the invention and treating an individual having such a predisposition or susceptibility to prevent or lessen osteoarthritis.

Known therapies for osteoarthritis can be effective in halting advancement of the disease, or at least slowing the advancement. Such existing therapies are generally not, however, able to bring about a dramatic reversal of the disease state, that is to say it are not capable of inducing a significant lessening of pain and discomfort for sufferers. It is thus an advantage of the invention that early diagnosis of osteoarthritis is improved, so that preventative therapy can be started. As alternative diagnostic methods improve and are developed, so the invention can add to the total knowledge of the risk of developing osteoarthritis of an individual. The decision of a physician on how and whether to initiate therapy in anticipation of the disease can be taken with increased confidence.

Suitable treatments for use in the invention comprise prescribing osteoarthritis preventative or palliative therapy, such as exercise to keep joints flexible, exercise to

- 7 -

improve muscle strength, use of joint protection to prevent strain or stress on joints, weight control to prevent stress on weight bearing joints and surgery to prevent or control joint stress. Suitable pharmaceutical treatments include the use of symptom modifying agents or disease modifying agents.

A variety of suitable treatments of osteoarthritis are described in the art, and the contents of these are incorporated herein by reference. WO-A-97/00675 describes the use of anthraquinone compounds for treatment of osteoarthritis, and US-A-5591740 describes treatment of osteoarthritis using 3,4-azepinyl-pyrrole derivatives. A dermatological preparation for treatment of osteoarthritis is described in JP-A-08/169832. WO-A-96/18403 describes a further composition for treatment of osteoarthritis, comprising diclofenac sodium and trebenoside. Other pharmaceutical treatments for osteoarthritis are described in WO-A-96/09043 and JP-A-08/053403. A medial collateral ligament brace for medical treatment of knee ligament damage and osteoarthritis is described in US-A-5562605. Other treatments will be known to persons of skill in the art.

A third aspect of the invention provides a composition for use in diagnosing a disease associated with a genetic polymorphism in a vitamin D receptor gene in an animal predisposed or susceptible to said disease, said composition comprising (a) one or more primer nucleic acid molecules adapted to amplify a portion of a vitamin D receptor gene selected from (i) a portion of exon 9 and (ii) a portion of the gene between exon 7 and the 3' UTR, and (b) means for identifying a polymorphism in (i) exon 9 or (ii) a portion of the gene from exon 7 to the 3' UTR.

A third aspect of the invention also provides a composition for use in identifying an animal predisposed or susceptible to a disease associated with a genetic polymorphism in a vitamin D receptor gene, said compositions comprising (a) one or more primer nucleic acid molecules adapted to amplify a portion of the vitamin D receptor gene selected from (i) a portion of exon 9 and (ii) a portion between exon 7 and the 3' UTR, and (b) means for identifying a polymorphism in (i) exon 9 or (ii) a portion of the gene from exon 7 to the 3' UTR.

- 8 -

In an embodiment of the invention, the composition of the third aspect of the invention comprises a nucleic acid molecule capable of identifying a polymorphism in said vitamin D receptor gene, said polymorphism being indicative of a risk genotype in said animal.

A further embodiment of the third aspect of the invention provides a composition for diagnosis of predisposition or susceptibility to osteoarthritis, comprising means for determining genotype of a vitamin D receptor gene of an individual, for example whether an individual is homozygous or heterozygous for polymorphic variants of a vitamin D receptor gene.

In an embodiment of the invention a diagnostic composition comprises PCR primers adapted to amplify a DNA sequence within exon 9 of a vitamin D receptor.

A diagnostic composition according to a further embodiment of the invention comprises PCR primers adapted to amplify a DNA sequence within exon 9 of a vitamin D receptor gene, wherein alternative versions of the gene are distinguished one from another by whether said sequence contains or lacks a site for cleavage by restriction enzyme TaqI. Suitable PCR primers are

- 1: CAGAGCATGGACAGGGAGCAAG (3')
- 2: GCAACTCCTCATGGCTGAGGTCTC (3')

Additionally, the diagnostic composition according to the invention can contain a supply of restriction enzyme TaqI.

In a fourth aspect of the invention there is provided a diagnostic kit comprising a diagnostic composition as described above and an indicator composition for indicating how possessing a polymorphic version of a vitamin D receptor gene correlates with predisposition or susceptibility to osteoarthritis.

Diagnostic kits are typically accompanied by or comprise a chart or other visual aid

- 9 -

for assistance in interpreting the results obtained using the kit. Suitable indicator compositions for use in the diagnostic kit of the invention include a leaflet or other visual reminder that possessing the risk polymorphism version of a vitamin D receptor gene correlates with increased risk of predisposition or susceptibility to osteoarthritis.

In a still further aspect of the invention there is provided use, in the manufacture of means for diagnosing whether an individual has a predisposition or susceptibility to osteoarthritis, of PCR primers adapted to amplify a region of exon 9 in a vitamin D receptor gene. Alternative versions of the gene are typically distinguished one from another by whether said region contains a site of cleavage by TaqI. Suitable PCR primers are

1: CAGAGCATGGACAGGGAGCAAG (3')

2: GCAACTCCTCATGGCTGAGGTCTC (3')

According to the invention, an individual who is homozygous for a risk polymorphism, that is to say homozygous for a version of the gene which is free of a TaqI cleavage site in exon 9, is classified as being at highest risk. An individual being heterozygous is classified as having moderate risk.

Optionally, the assessment of an individual's risk factor according to any aspect of the invention is calculated by determining the genotype of a vitamin D receptor gene polymorphism and combining the result with analysis of other known genetic or physiological or dietary or other indications. The invention in this way provides further information on which measurement of an individual's risk can be based.

In exercise of a specific embodiment of the invention, described in detail below, an association has been demonstrated between a vitamin D receptor gene polymorphism and early knee osteoarthritis in women from the general population. In the specific embodiment of the invention, the risk polymorphism has been indicated as T, and absence of the risk polymorphism as t. The TT genotype is over represented in women with knee osteoarthritis compared to the tt genotype and appears to offer a 4-

- 10 -

fold risk of development of this disease state when compared to the alternate homozygous genotype tt. This relationship can not be explained on the basis of age, as no significant deviation from expected genotype frequencies in the total group after stratification by age was observed. A secular alteration in population structure due to either migration or immigration in the 1930's-40's or premature death which could both have altered the genotype frequencies is therefore unlikely.

Although knee osteoarthritis has been suspected to be under genetic influence, no candidate genes have hitherto been reported. The vitamin D receptor locus maps to chromosome 12q12-14 by somatic cell hybridisation. The TaqI polymorphism represents a synonymous codon change in exon 9 of the vitamin D receptor gene, and therefore does not alter the receptor's protein structure. It is possible that vitamin D receptor polymorphisms are not the disease causing genes but are instead in linkage disequilibrium with a nearby novel disease susceptibility gene on chromosome 12. Nevertheless, the observed correlation is of use in diagnosis of risk of predisposition or susceptibility to osteoarthritis.

There now follows description of a specific embodiment of the invention.

Patients and Methods

The study design was a nested case-control study within a population cohort of 1003 women, with a mean age (\pm SD) of 54.2 ± 6.0 years. Women in the age range 45-64 had been selected from a large single general practice in Chingford, North-East London (total of 11,000 registered patients) to participate in a longitudinal epidemiological study of rheumatic diseases. 1,353 women were found to be in the age range specified, and of these 78% (1,003) agreed to participate. The area is predominantly middle class, 98% are white and the population similar to UK normals in terms of height, weight, smoking status, hysterectomy rates and use of hormone replacement therapy (HRT) (Hart DJ, Spector TD: The relationship of obesity, fat distribution and osteoarthritis in the general population: the Chingford Study. *J Rheumatol* 20:331-335, 1993). Women were selected for this study on the basis of

- 11 -

postmenopausal status, which was defined by absence of menstruation for 12 months and confirmed by measurement of sex hormones (serum estradiol, luteinising hormone and follicular stimulating hormone). All women completed an extensive questionnaire detailing risk factors for both osteoporosis and OA. The study was approved by the Local Ethics Committee and all women gave informed consent to participate.

Antero-posterior weight bearing knee radiographs were performed using standard procedures and subsequently graded blind by a single observer (DJH) according to the methods of Kellgren & Lawrence where a scale of 0 to 4 is used (0 representing no disease and 4 representing severe disease) (Kellgren JH, Lawrence JS: The Epidemiology of Chronic Rheumatism. Oxford: Blackwell Scientific, 1963). Radiological definition of OA is the currently accepted standard for epidemiological studies of OA in populations (Spector TD, Hart DJ, Byrne J, Harris PA, Dacre JE, Doyle DV: Definition of osteoarthritis of the knee for epidemiological studies. Ann Rheum Dis 52:790-794, 1993). Knee OA was defined as present if a grade of 2 or more was given, and controls were classified as having no OA with a grade of 1. The intra-observer reproducibility for this technique was good with a kappa score of over 0.8. Presence of Heberden's nodes was determined by clinical hand examination (Egger P, Cooper C, Hart DJ, Doyle DV, Coggon D, Spector TD: Patterns of joint involvement in osteoarthritis of the hand. J Rheumatol 22:1509-1513, 1995). BMD was measured at the femoral neck using dual energy xray absorptiometry with a Hologic QDR-1000 (Hologic Inc., Waltham, MA). Reproducibility (CV %), assessed by duplicate measures in healthy volunteers, was 1.4% at the femoral neck.

DNA was extracted from peripheral blood leucocytes using standard techniques. PCR was used to amplify a 740 bp fragment of the VDR gene using published primers and reaction conditions (Spector TD, Keen RW, Arden NK, Morrison NA, Major PJ, Nguyen TV, Baker JR, JR Baker, Sambrook PN, Lanchbury JS, Eisman JA: Vitamin D receptor gene (VDR) alleles and bone density in postmenopausal women: a UK twin study. BMJ 310: 1357-1360, 1995; Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA: Prediction of bone density from vitamin D receptor alleles. Nature 367: 284-287, 1994). VDR genotypes were obtained by

- 12 -

digestion of the PCR product with the restriction enzyme TaqI (Promega Corps) and alleles were coded as "T" (absence of TaqI restriction site) and "t" (presence of restriction site). Differences in demographic variables between OA cases and controls, and between VDR genotypes were initially compared using analysis of variance and chi-squared test. VDR genotype frequencies were compared between OA and control groups using Fishers exact test. Conditional logistic regression analysis was used to estimate the odds ratio and 95% test based confidence intervals for developing a radiological feature of OA for the individual VDR genotypes, with the homozygous VDR genotype "tt" set as baseline. Adjustment for other potential confounding variables was performed using conditional logistic regression with the PC software statistical programme STATA.

Results

In total, 501 women from the total cohort were postmenopausal and had graded knee radiographs. Full genotype results were available on 351 (70%) women: 82 subjects with knee OA and 269 with normal radiographs. The mean age \pm SD for the total group of 351 women was 55.3 ± 5.0 years. Subdivision of women with genotype results according to their radiological grading for OA showed that of the cases, 63 (76.8 %) were grade 2, 15 (18.3 %) grade 3 and 4 (4.9 %) grade 4. For controls, 201 (74.7 %) were grade 0 and 68 (25.3 %) grade 1. The characteristics of the women according to the absence or presence of knee OA are shown in Table 1, and are similar to those of the whole cohort except for age, menopause status and duration. No significant differences were observed between subjects with genotype results and those where DNA was unavailable. Within the 351 women with full results, there were significant differences between the cases and controls for potential confounders such as age, body mass index (BMI), use of HRT, and hip BMD.

VDR genotype frequencies in the total group of 351 women were similar to those reported in other Caucasian populations and in Hardy Weinberg equilibrium (Spector TD, Keen RW, Arden NK, Morrison NA, Major PJ, Nguyen TV, Baker JR, JR Baker, Sambrook PN, Lanchbury JS, Eisman JA: Vitamin D receptor gene (VDR) alleles and

- 13 -

bone density in postmenopausal women: a UK twin study. *BMJ* 310: 1357-1360, 1995; Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA: Prediction of bone density from vitamin D receptor alleles. *Nature* 367: 284-287, 1994). There were no significant differences in the baseline characteristics as measured in Table 1 between the VDR genotypes within this total group or after stratification by Kellgren and Lawrence OA grade. The frequencies of the TaqI genotypes were found to differ significantly between the OA cases and controls using Fisher's exact test ($P = 0.03$) with the tt genotype frequency being reduced with worsening OA status, although the number of subjects with severe OA (grade 3) was too small for formal analysis (Table 2). The frequency of the T allele was increased by 5% in the OA group compared to controls, although this difference was non-significant. The odds ratios (95% CI) for knee OA in the "TT" VDR genotype group compared to the alternate homozygous genotype "tt" was 2.42 (0.94-6.21, $p = 0.07$), and in the heterozygous genotype Tt 3.15 (1.26-7.83, $p = 0.01$) (Table 3). After adjustment for variables judged to be potential confounders (age, BMI, HRT use and hip BMD), the odds ratio for knee OA (95% CI) in those with the TT genotype (TT vs tt) remained marginally increased at 2.82 (0.98-8.10, $p < 0.05$). After similar adjustment the odds ratio for the "Tt" genotype (Tt vs tt) was marginally reduced at 2.98 (1.09-8.12, $p = 0.03$). As risk of knee OA appeared to be associated with presence of the T allele with a dominant inheritance pattern, analysis was subsequently performed on the genotypes TT and Tt combined against the tt homozygous group set at baseline. Crude analysis showed an odds ratio of 2.82 (1.16-6.85, $p = 0.02$) for risk of knee OA in association with the T allele. This increase in risk remained after adjustment as previously detailed with an odds ratio of 2.60 (1.01-6.71, $p < 0.05$).

No significant relationship was observed between VDR genotype and nodal arthritis, as determined by the presence of Heberden's nodes. In addition there was no difference in genotype frequencies in the subset of knee OA cases who also had clinical nodes present compared to knee OA alone (data not shown). Within the total group of 351 women we were also not able to demonstrate any overall significant relationship between BMD and VDR genotype, although there was a 4.6 % difference

- 14 -

in BMD at the hip between the homozygous genotypes TT and tt. Adjustment for potential confounders such as age, BMI, HRT use and OA grade did not appreciably alter these findings.

Discussion

Our data demonstrate an association between a TaqI polymorphism of the VDR locus and early knee OA in women from the general population. The "T" allele is associated with a nearly 3-fold increased risk for development of knee OA when compared to the alternate allele. The pattern of risk was observed to be co-dominant, with the homozygous genotype TT and the heterozygous genotype Tt both having an equivalent increased risk of knee OA. This relationship cannot be explained on the basis of age, as we observed no significant deviation from expected genotype frequencies in the total group after stratification by age. This relationship was also independent of other factors such as BMI, HRT use and bone mineral density which have been shown to influence OA risk and were found to differ between the cases and controls. Our finding that there was no relationship between the VDR locus and nodal arthritis suggests that differing genetic mechanisms may underlie the development of Heberdens nodes and associated generalised osteoarthritis.

The invention thus provides method and apparatus for diagnosis and treatment of individuals having a predisposition or susceptibility to osteoarthritis, by determining the genotype of the individual in respect of the vitamin D receptor gene. Early diagnosis or improved diagnosis enables an individual to receive preventative or palliative treatment at a time when this treatment can be effective.

Table 1

Demographic Variables of Women and their Knee OA Status (mean \pm SD unless indicated)

Variable	Controls			OA Cases			
	Total (n=269)	Grade 0 (n=201)	Grade 1 (n=68)	Total (n=82)	Grade 2 (n=63)	Grade 3 (n=15)	Grade 4 (n=4)
Age (yrs)	54.4 (4.8)	54.1 (4.8)	55.4 (4.6)	58.3* (4.7)	58.1 (4.6)	58.1 (5.2)	62.5 (1.9)
BMI (kg/m ²)	25.0 (3.6)	24.5 (3.5)	26.2 (3.8)	28.0* (4.0)	27.7 (3.7)	28.4 (4.5)	30.8 (4.9)
Age at menopause (yrs)	47.8 (5.0)	47.9 (4.9)	47.1 (5.3)	47.7 (4.7)	47.7 (4.8)	48.1 (4.8)	46.5 (4.1)
Ever use of HRT (n, %)	78 (29%)	57 (28%)	21 (31%)	15 † (18%)	10 (16%)	5 (33%)	0 (0%)
Ever smokers (n, %)	112 (42%)	83 (41%)	29 (43%)	37 (45%)	29 (46%)	6 (40%)	2 (50%)
BMD Femoral neck (g/cm ²)	0.73 (0.12)	0.73 (0.11)	0.75 (0.12)	0.77 ‡ (0.13)	0.77 (0.14)	0.78 (0.10)	0.72 (0.08)

*P < 0.001 (cases vs controls)

†P = 0.05 (cases vs controls)

‡P = 0.008 (cases vs controls)

- 16 -

Table 2

VDR Genotype Frequencies (n, %) according to Kellgren and Lawrence OA Grade

	Grade 0 (n = 201)	Grade 1 (n = 68)	Grade 2 (n = 63)	Grade 3 (n = 15)	Grade 4 (n = 4)
TT	74 (36.8 %)	24 (35.3 %)	19 (30.2 %)	10 (66.7 %)	0 (0 %)
Tt	87 (43.3 %)	36 (52.9 %)	38 (60.3 %)	4 (26.7 %)	4 (100 %)
tt	40 (19.9 %)	8 (11.8 %)	6 (9.5 %)	1 (6.6 %)	0 (0 %)

Table 3

Effect of VDR Genotype on Risk of Knee OA - Odds Ratio (95% Confidence Interval) Crude and Adjusted

VDR Genotype	Frequency in OA cases (%)	Frequency in controls (%)	OR (Crude)	OR (Adjusted *)
TT	29 (35.4 %)	98 (36.4 %)	2.42 (0.94, 6.21)	2.82 (0.98, 8.10)
Tt	46 (56.1 %)	123 (45.7 %)	3.15 (1.26, 7.83)	2.98 (1.09, 8.12)
tt	7 (8.5 %)	48 (17.9 %)	1.0	1.0

* Adjusted for age, BMI, femoral neck BMD, use of HRT

CLAIMS

1. A method of diagnosing a disease associated with a genetic polymorphism in a vitamin D receptor gene in an animal predisposed or susceptible to said disease, said method comprising determining the genotype of said vitamin D receptor gene in said animal.
2. A method of identifying an animal predisposed or susceptible to a disease associated with a genetic polymorphism in a vitamin D receptor gene, said method comprising determining the genotype of said vitamin D receptor gene in said animal.
3. The method of Claim 1 or Claim 2 comprising determining whether an individual is homozygous or heterozygous for alternative versions of a vitamin D receptor gene.
4. The method of Claim 3 comprising determining whether a fragment of the vitamin D receptor gene contains a cleavage site for a restriction enzyme selected from TaqI, BsmI and Apol.
5. The method of Claim 4 comprising amplifying a fragment of exon 9 of a vitamin D receptor gene or a region from exon 7 to the 3' UTR of the gene.
6. The method of Claim 5 further comprising classifying an individual according to whether the fragment contains a cleavage site for TaqI, wherein possessing a fragment that lacks the cleavage site correlates with increased risk of predisposition or susceptibility to osteoarthritis or whether the region contains a cleavage site for BsmI or Apol, wherein possessing a region that contains the cleavage site correlates with increased risk.

7. The method of Claim 6 wherein the fragment is amplified using the PCR primers:

1: CAGAGCATGGACAGGGAGCAAG (3')

2: GCAACTCCTCATGGCTGAGGTCTC (3')

8. A method of diagnosis of predisposition or susceptibility to osteoarthritis in an individual, the method comprising determining whether the individual possesses a polymorphic risk version of a vitamin D receptor gene, a polymorphic risk version of the gene being one that does not contain a cleavage site for restriction enzyme TaqI, the method comprising

using PCR techniques to amplify a fragment of exon 9 of the vitamin D receptor gene to make copies of the fragment,

testing whether the copies contain a cleavage site for TaqI,

and thereby determining whether the individual is homozygous or heterozygous for a polymorphic risk version of the gene, and

diagnosing that individual's risk of predisposition or susceptibility to osteoarthritis as greatest if that individual is homozygous for the polymorphic risk version of the gene and least if that individual lacks the polymorphic risk version.

9. The method of Claim 8 comprising using the PCR primers:

1: CAGAGCATGGACAGGGAGCAAG (3')

2: GCAACTCCTCATGGCTGAGGTCTC (3')

to amplify a 740 base pair fragment of exon 9 of a vitamin D receptor gene.

10. A method of treating an animal predisposed or susceptible to osteoarthritis, said method comprising:-

- (a) determining the genotype of a vitamin D receptor gene in said animal to identify a risk genotype in said vitamin D receptor gene; and
- (b) administering to said animal an effective dose of a therapeutic composition suitable to delay, reduce or prevent osteoarthritis in said animal.

11. The method of Claim 10, comprising administering to said animal an effective dose of a therapeutic composition selected from the group consisting of anthraquinone compound, 3,4-azepinyl-pyrrole derivatives, prosterglandins, compositions comprising diclofenac sodium and trebenoside, compliment inhibitors, collagenase inhibitors and osteogenic promoters.

12. A method of treating an animal predisposed or susceptible to osteoarthritis, said method comprising:-

- (a) determining the genotype of a vitamin D receptor gene in said animal to identify a risk genotype in said gene; and
- (b) carrying out or prescribing osteoarthritis preventative or palliative therapy.

13. The method of Claim 12 comprising prescribing for carrying out osteoarthritis preventative or palliative therapy, selected from the group consisting of exercise to keep joints flexible, exercise to improve muscle strength, use of joint protection to prevent strain or stress on joints, weight control to prevent stress on weight bearing joints and surgery to prevent or control joint stress.

- 20 -

14. The method of Claim 10 or Claim 12 comprising determining genotype of exon 9 of said vitamin D receptor gene, or of a region from exon 7 to the 3' UTR.
15. The method according to Claim 14 wherein said animal is a human.
16. A composition for use in diagnosing a disease associated with a genetic polymorphism in a vitamin D receptor gene in an animal predisposed or susceptible to said disease, said composition comprising (a) one or more primer nucleic acid molecules adapted to amplify a portion of exon 9 of a vitamin D receptor gene in said mammal or a region from exon 7 to the 3' UTR, and (b) means for distinguish between polymorphisms of said amplified portion.
17. The composition of Claim 16 comprising PCR primers adapted to amplify a DNA sequence within exon 9 of a vitamin D receptor gene, wherein alternative versions of the gene are distinguished one from another by whether said sequence contains or lacks a site for cleavage by restriction enzyme TaqI.
18. The composition of Claim 16 or 17 comprising the PCR primers
- 1: CAGAGCATGGACAGGGAGCAAG (3')
- 2: GCAACTCCTCATGGCTGAGGTCTC (3')
19. The composition of Claim 18 further comprising a supply of restriction enzyme TaqI.
20. The composition of Claim 16 further comprises a supply of restriction enzyme BsmI or ApeI.
21. Use of PCR primers adapted to amplify a fragment of exon 9 of a vitamin D

- 21 -

receptor gene, in manufacture of a composition for diagnosis of predisposition or susceptibility to osteoarthritis.

22. Use of PCR primers adapted to amplify a region of the vitamin D receptor gene between exon 7 and the 3' UTR, in manufacture of a composition for diagnosis of predisposition or susceptibility to osteoarthritis.

PCT/GB 97/01107

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/GB 97/01107

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 15, 1 August 1992, pages 6665-6669, XP000569585 MORRISON N A ET AL: "CONTRIBUTION OF TRANS-ACTING FACTOR ALLELES TO NORMAL PHYSIOLOGICAL VARIABILITY: VITAMIN D RECEPTOR GENE POLYMORPHISMS AND CIRCULATING OSTEOCALCIN" see the whole document ---	1-5
X	NATURE, vol. 367, - 20 January 1994 pages 284-287, XP002036961 MORRISON N. A. ET AL.,: "Prediction of bone density from vitamin D receptor alleles" cited in the application see the whole document ---	1-5
Y	WO 95 31722 A (LIGAND PHARM INC) 23 November 1995 see claims ---	6-9
Y	EP 0 435 362 A (UNIV NEW YORK) 3 July 1991 see page 4, lines 25 ff; claims ---	11
Y	WILSON ET AL.,: "Harrison's principles of internal medicine" 1991, MCGRAW-HILL XP002036962 see page 1475 - page 1479 ---	11
P,X	BRITISH JOURNAL OF RHEUMATOLOGY; ABSTRACT SUPPL. 1, vol. 35, - 8 May 1996 page 117 XP002037350 GRIFFITHS G.O. ET AL.,: "Polymorphisms of the vitamin D receptor and osteoarthritis" see the whole document -----	1-6,8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 97/01107

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 10-11
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 10 to 11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.: 12-15
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
See PCT/Rule 39.1(iv).
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

PCT/GB 97/01107

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9403633 A	17-02-94	AU 673187 B AU 4690093 A CA 2141300 A EP 0652975 A JP 8501933 T US 5593833 A	31-10-96 03-03-94 17-02-94 17-05-95 05-03-96 14-01-97
WO 9531722 A	23-11-95	AU 2646295 A CA 2190275 A EP 0765475 A	05-12-95 23-11-95 02-04-97
EP 0435362 A	03-07-91	CA 2031368 A ES 2055309 T JP 3227931 A US 5459135 A US 5321017 A US 5308839 A	05-06-91 16-08-94 08-10-91 17-10-95 14-06-94 03-05-94